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Etiologic factors associated with p53 immunostaining in cutaneous malignant melanoma

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Findings from a case-control study of cutaneous malignant melanoma (CMM) in Queensland, Australia, suggest that melanomas exhibiting p53 immunostaining possess different risk factors from those of other melanomas. To further explore this hypothesis, a case-only analysis of risk factors for p53 immunostaining with anti-p53 MAb DO-7 was undertaken in 523 people diagnosed with CMM in Canada and Australia. Phenotypic factors and past sun exposure were measured using a self-administered questionnaire and telephone interview. The presence of strong p53 staining (>10% of cell nuclei positively stained *vs.* <1% staining) was positively associated with some indicators of high cumulative sun exposure: lentigo maligna melanoma subtype (OR = 3.2 *vs.* superficial spreading subtype), melanoma location on the head and neck (OR = 2.8 *vs.* back), histopathologic evidence of solar elastosis (OR = 2.1) and previous diagnosis of nonmelanoma skin cancer (OR = 2.4). Strong staining was negatively associated with high nevus density on the back (OR = 0.2 for >25 nevi *vs.* 0–3 nevi) and histologic evidence of a coexisting nevus (OR = 0.3). Other factors associated with strong p53 immunostaining include greater Breslow thickness (OR = 7.4 for >4.00 *vs.* <0.76 mm), male sex (OR = 2.2) and dense freckling (OR = 6.6 *vs.* few freckles). Of these, thickness, male sex, dense freckling, low nevus density on the back, histologic subtype and history of nonmelanoma skin cancer appeared to be independently associated with strong p53 staining. Our findings are consistent with the Queensland study in suggesting that variables indicating high accumulated sun exposure are positively associated with p53 staining and that an increased number of nevi is positively associated with its absence; they may reflect etiologic and pathogenetic heterogeneity in melanoma.
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Key words: melanoma; p53; risk factor; etiology; epidemiology; case-only study

There is indirect evidence for the hypothesis of etiologic heterogeneity in melanoma. Melanomas occurring on habitually sun-exposed body sites differ from lesions on intermittently exposed sites in their distribution of histologic subtypes (LMM usually located on head and neck, superficial spreading and nodular melanomas more commonly found on trunk for men and legs for women)[1–3] and age at diagnosis (melanomas on face showing a near exponential increase in incidence with age, while incidence on other areas is highest in middle age and falls thereafter).[2,4] As well, melanomas on intermittently exposed body sites are commonly associated with a nevus, while this finding is uncommon for melanomas arising on the head and neck.[5–8] Case-control studies investigating risk factors for melanomas arising on different body sites have also suggested etiologic heterogeneity.[5,7,9–12]

In a small case-control study of melanoma, Whiteman *et al.*[13] investigated the etiologic correlates of abnormal tumor cell levels of p53, a nuclear protein that plays a critical role in safeguarding the genome through regulation of cell division, DNA repair and apoptosis. Melanomas with abnormally high nuclear levels of p53 were associated with measures of high cumulative sun exposure and sun-sensitive pigmentation phenotype, while immunonegative tumors were associated with increased nevus density and freckling. These findings led the investigators to propose a "divergent pathway" model for sporadic melanoma induction, whereby high cumulative sun exposure is necessary for promoting clonal expansion and driving tumor progression for some melanomas (characterized by p53 overexpression), while endogenous factors associated with a high propensity for melanocyte proliferation (characterized by high nevus density) are sufficient to drive the promotion and progression of other tumors.

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Abbreviations: BC, British Columbia; CI, confidence interval; CMM, cutaneous malignant melanoma; GEM, International Study of Genes, Environment and Melanoma; LMM, lentigo maligna melanoma; MAb, monoclonal antibody; NSW, New South Wales; OR, odds ratio; TBS, TRIS-buffered saline.

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The interpretation of these findings, however, is limited by imprecision due to the small number of immunostained cases ($n = 121$). Immunohistochemical studies have reported 0–66% of melanomas to have abnormally high tumor cell levels of p53.[14] Evidence of such immunostaining is often taken to suggest *TP53* mutation since mutant p53 frequently has a longer half-life than the wild-type protein and can be detected using labeled anti-p53 antibodies. However, *TP53* mutations have been rarely found in uncultured primary melanomas.[14]

To evaluate whether melanomas characterized by the presence or absence of abnormal p53 levels may arise through separate causal pathways, we carried out a case-only analysis using subjects enrolled in a multicenter international study investigating the genetic epidemiology of melanoma, the Genes Environment and Melanoma (GEM) study.

TABLE I – COMPARISON OF p53 STAINING SCORES FOLLOWING REREVIEW OF 42 TUMORS

Original review	Reliability re-review		
	No staining (<1% of cells positive)	Weak staining (1–10% of cells positive)	Strong staining (>10% of cells positive)
No staining (<1% of cells positive)	20	9	0
Weak staining (1–10% of cells positive)	2	5	3
Strong staining (>10% of cells positive)	0	0	3

Agreement for 3-level p53 score: simple κ 0.39 (0.12–0.63); weighted κ 0.51 (0.29–0.73). Agreement for 2-level (≥1% *vs.* <1%) p53 score: simple κ 0.47 (0.21–0.72). Agreement for 2-level (>10% *vs.* ≤10%) p53 score: simple κ 0.63 (0.26–1.00).

Material and methods

Study population

GEM is a collaborative project involving 9 centers in 4 countries (Australia, Canada, Italy and the United States) investigating interaction of sun exposure, pigmentation phenotype and genes involved in cell-cycle control (*CDKN2A*), melanin synthesis (*MC1R*) and DNA repair (selected nucleotide excision repair genes). In this case-control study, subjects with 2 or more primary malignant melanomas are compared to controls with a first primary only.[15]

For our case-only analysis, we initially drew cases from GEM controls in Ontario and BC, Canada. Eligibility criteria included age 18 years or older and date of diagnosis of a first primary invasive melanoma between January 1 and June 30, 2000 (August 30, 2000, for Ontario). Subjects diagnosed with acral lentiginous melanoma were excluded, as were individuals who could not complete the telephone interview for reasons of cognitive or language difficulty. Archived tissue from the tumors of all eligible GEM participants from Ontario and BC was requested to assess p53 immunopositivity. To increase the power of our analysis, our case series was expanded to include tumor specimens from 100 eligible controls enrolled by the GEM study center in NSW, Australia. This sample was randomly selected from specimens of Breslow thickness ≤2.00 mm, which were being reviewed by our study dermatopathologist as part of a separate case-only project, and restricted to specimens located in laboratories with 10 or more GEM specimens. The restriction on laboratory size was used to reduce the time taken for and cost of obtaining specimens.

Our study was approved by the local Institutional Review Boards of the University of Toronto, the BC Cancer Agency, the University of BC and the Cancer Council of NSW.

Data collection

Eligible cases of first primary invasive melanoma were ascertained from pathology reports received by the Ontario, BC and NSW population-based cancer registries. Physicians caring for eligible cases were contacted by study staff for permission to approach their patients and (in Ontario and BC) for patient contact information.

The same data collection instruments were used in each study center. A self-administered mailed questionnaire sought information on pigmentation phenotype and nevus density and on residence and occupation at each decade year of life (*i.e.,* at ages 10, 20 and up to the most recent completed decade year). Ontario and BC subjects returned signed study consent forms along with these questionnaires. In NSW, subjects were mailed a letter of invitation, and consent was sought prior to sending them the questionnaire or other study material.

A computer-assisted telephone interview collected information on lifetime exposure to sunlight, family history of melanoma and other cancers, sun sensitivity phenotype and demographic characteristics. This telephone interview was adapted from the interview instrument used in the Geraldton Skin Cancer Prevention Survey.[16] The Geraldton instrument was developed to improve the accuracy of recalled lifetime sun exposure through the inclusion of a personal calendar in which subjects recorded residences and jobs in each year of life. It has shown high test–retest reliability,[17] and an adapted version has been used successfully in a study of sun exposure and ocular melanoma.[18] The information from the calendar was used as memory cues for a series of structured questions on personal sun exposure during outdoor activities and other sun-related behaviors at specific ages. The telephone interview took between 25 and 60 min to complete.

Histopathologic review

Slides from each melanoma were reviewed by the study dermatopathologist (L.F.) to record histopathologic characteristics of the tumors, including evidence of a coexisting nevus. Whenever the original diagnostic slides were not available, recuts were reviewed.

Immunohistochemistry

The study dermatopathologist selected a representative block to be cut when reviewing the slides. Tumor sections of approximately 5 μm thickness were cut from each block and mounted on charged slides.

The p53 MAb (clone DO-7, 1:500 dilution; Novocastra, Newcastle-upon-Tyne, UK) was applied to paraffin sections of tumors according to standard immunohistochemical techniques, with inclusion of a microwave epitope retrieval step to improve sensitivity.[19] Sections were deparaffinized in xylene and rehydrated through a series of graded alcohols. Endogenous peroxidase activity of the sections was quenched using 3% hydrogen peroxide. Heat-induced antigen retrieval was performed using a microwave pressure cooker. After simmering for 10 min, sections were removed and cooled at room temperature for 30 min, then rinsed and incubated with 10% nonimmune goat serum for 10 min. Slides were subsequently incubated with DO-7 for 1 hr at room temperature. Sections were washed and covered with biotinylated secondary antibody for 15 min. After washing with PBS 3 times, sections were covered in peroxidase-conjugated streptavidin for 15 min and then washed 3 times with TBS before applying the enzyme substrate 9-amino 3-ethylcarbazole, which produced a red color. Sections were counterstained with freshly filtered Mayer's hematoxylin and covered with aqueous mountant (Crystal Mount, Biomedica, CA).

With each batch of slides, a section from a colon cancer specimen known to exhibit strong staining for p53 was stained in an identical manner and served as a positive control. As a negative control, normal tissue adjacent to the colorectal tumor known not to stain for p53 was examined for evidence of staining.

All slides were viewed under a microscope by both study pathologists (L.F. and H.K.) and scored for positive staining (only red nuclear staining in melanoma cells was considered evidence of positive staining). The proportion of positively staining cells in

TABLE II – ASSOCIATIONS BETWEEN DEMOGRAPHIC AND HISTOPATHOLOGIC CHARACTERISTICS AND EVIDENCE OF p53 IMMUNOSTAINING (1–10%, >10% TUMOR CELL NUCLEI POSITIVE *VS.* <1% POSITIVE) IN CMM (n = 523)

Characteristic	Cell nuclei positive [n (%)][1]			$OR_{1-10\%}$[2,3]	(95% CI)	$OR_{>10\%}$[2,4]	(95% CI)
	<1%	1–10%	>10%				
Study center							
Ontario	245 (73)	66 (20)	24 (7)	1.0		1.0	
BC	57 (63)	23 (26)	10 (11)	1.5	(0.9–2.6)	1.9	(0.9–4.2)
NSW	87 (89)	8 (8)	3 (3)	0.3*	(0.2–0.7)	0.3	(0.1–1.2)
Age (years)							
<40	58 (71)	18 (22)	6 (7)	1.0		1.0	
40–54	126 (75)	33 (20)	8 (5)	0.9	(0.4–1.7)	0.5	(0.2–1.5)
55–69	116 (73)	28 (18)	15 (9)	0.8	(0.4–1.5)	0.9	(0.3–2.7)
70+	89 (77)	18 (16)	8 (7)	0.7	(0.3–1.4)	0.7	(0.2–2.1)
p_{trend}				*0.27*		*0.99*	
Sex							
Female	193 (77)	46 (18)	11 (4)	1.0		1.0	
Male	196 (72)	51 (19)	26 (10)	1.2	(0.7–1.9)	2.2*	(1.1–4.8)
Breslow thickness (mm)							
<0.76	216 (81)	36 (14)	14 (5)	1.0		1.0	
0.76–1.50	119 (77)	27 (17)	9 (6)	1.3	(0.8–2.3)	1.2	(0.5–2.9)
1.5–4.00	44 (54)	29 (36)	8 (10)	3.7*	(2.0–6.7)	2.5	(1.0–6.3)
>4.00	9 (47)	5 (26)	5 (26)	2.9	(0.9–9.2)	7.4*	(2.1–25.7)
p_{trend}				*0.0004*		*0.0003*	
Histologic subtype							
SSM	287 (77)	71 (19)	14 (4)	1.0		1.0	
NM	38 (64)	15 (25)	6 (10)	0.7[5]	(0.3–1.5)	1.9[5]	(0.6–5.8)
LMM	33 (72)	8 (17)	5 (11)	1.1[5]	(0.5–2.8)	3.2[5]	(1.0–10.4)
Other, NOS	30 (68)	3 (7)	11 (25)	0.2[5]*	(0.1–0.7)	4.8[5]*	(1.8–12.6)
Evidence of a coexisting nevus							
Absent	235 (72)	63 (19)	28 (9)	1.0		1.0	
Present	147 (79)	34 (18)	4 (2)	0.9[5]	(0.6–1.5)	0.3[5]*	(0.1–0.8)

NOS, not otherwise specified; SSM, superficial spreading melanoma; NM, modular melanoma; LMM, lentigo maligna melanoma.–[1]Counts may not sum to the total number of study subjects due to missing data.–[2]ORs adjusted for study center, sex and age group (modeled as continuous variable).–[3]$OR_{1-10\%}$ = OR for 1–10% of tumor cells positively stained *vs.* <1% positively stained.–[4]$OR_{>10\%}$ = OR for >10% of tumor cells positively stained *vs.* <1% positively stained.–[5]Also adjusted for Breslow thickness (modeled as continuous variable for $OR_{1-10\%}$ and $OR_{>10\%}$).–*$p < 0.05$.

each tumor was classified in one of 4 categories (<1%, 1–10%, 11–30% and >30% of tumor cell nuclei positive). This scoring system replicated that described by Whiteman *et al.*[13] A systematic 10% subsample of Ontario and BC specimens (n = 42) was rescored to assess intramethod reliability. The same immunostained slides were used as for the original scoring.

Data analysis

Putative predictors of p53 immunostaining were assessed from among measures of pigmentation phenotype (ethnicity, skin, hair and eye color, skin propensity to burn, skin tendency to tan, freckling as a child, nevus density), tumor characteristics (histologic subtype, body site, lesion thickness, Clark level) and sun exposure. An estimate of the total hours of sun exposure experienced during decade years was calculated from data collected from the telephone interview. Other measures estimating the amounts of different patterns of sun exposure were also calculated. Total sun exposure on working days was estimated as an indicator of the amount of more continuous, probably occupationally related exposure, while sun exposure on nonworking days was calculated as an estimate of the amount of intermittent-type sun exposure. Information on the frequency with which the melanoma site was covered when outdoors was used to calculate an estimate of total hours of sun exposure received at the melanoma site. Also included in the analysis were a history of sunburn at the site of the melanoma (both any sunburns and blistering sunburns only) and the number of vacations to sunny places. Two biologic markers of high cumulative sun exposure (previous diagnosis of nonmelanoma skin cancer and solar elastosis in the tissue adjacent to the melanoma) were also included in the analysis.

All data analyses were performed using SAS software (SAS Institute, Cary, NC; version 8.02). Data were edited before analysis to identify missing values, errors and outliers; corrections were made whenever possible. ORs with accompanying 95% CIs were calculated using maximum-likelihood estimates from polychotomous (multinominal) logistic regression models to describe the association of each variable with weak (1–10% positively stained) and strong (>10% positively stained) p53 staining with reference to <1% staining.

Continuous and ordinal variables were categorized for the analysis, to enable visualization of any nonlinear trends. Continuous variables other than age were categorized into quarters of distribution. Linear trend was analyzed using the Wald test by modeling each variable as a single continuous covariate. For continuous variables, the trend test was performed, using the median value within each category as the category score. Unless otherwise specified, all tests of statistical significance employed an α level of 0.05.

Adjusted ORs were calculated, controlling for age, sex and study center. To identify independent predictors of p53 immunostaining, multivariable models of p53 immunostaining were developed using stepwise ($p < 0.20$, 0.10 for entering and remaining in the model, respectively) and backward elimination ($p < 0.10$ for staying in the model) variable selection algorithms.

Tests of 2-way interaction with sun-exposure variables were performed for country of residence and tendency to tan upon repeated sun exposure (1, dark or moderate tan; 0, mild or no tan) using the likelihood ratio test. A reanalysis excluding LMMs was also done.

Results

In Ontario and BC, there were 934 eligible controls ascertained for the GEM study. Of these, 518 participated in GEM (participation rate: Ontario, 55%; BC, 58%). Archived tumor tissue was obtained for the lesions of 435 (84%) of these participants. In NSW, 1,150 eligible controls were ascertained for GEM, of whom 725 participated (63% participation rate). Two of the 100 NSW controls randomly selected for this study were subsequently found

TABLE III – ASSOCIATIONS BETWEEN PIGMENTATION PHENOTYPE FACTORS AND EVIDENCE OF p53 IMMUNOSTAINING (1–10%, >10% TUMOR CELL NUCLEI POSITIVE *VS.* <1% POSITIVE) IN CCM (n = 523)

Characteristic	Percentage of cell nuclei positive [n (%)][1]			$OR_{1–10\%}$[2,3]	(95% CI)	$OR_{>10\%}$[2,4]	(95% CI)
	<1%	1–10%	>10%				
Nevus density							
No nevi	97 (78)	20 (16)	7 (6)	1.0		1.0	
Low	202 (72)	53 (19)	25 (9)	1.2	(0.7–2.1)	1.6	(0.7–4.0)
Moderate or high	83 (75)	23 (21)	4 (4)	1.1	(0.6–2.3)	0.6	(0.2–2.1)
p_{trend}				*0.71*		*0.50*	
Nevi on back							
0–3	109 (73)	27 (18)	13 (9)	1.0		1.0	
4–10	88 (72)	27 (22)	8 (7)	1.3	(0.7–2.3)	0.8	(0.3–2.1)
11–25	88 (73)	20 (17)	13 (11)	0.8	(0.4–1.6)	1.1	(0.5–2.6)
>25	100 (79)	23 (18)	3 (2)	0.7	(0.4–1.3)	0.2*	(0.1–0.7)
p_{trend}				*0.19*		*0.01*	
Facial freckles at age 10							
Few	274 (274)	63 (17)	24 (7)	1.0		1.0	
Moderate	98 (73)	29 (22)	7 (5)	1.2	(0.7–2.0)	0.8	(0.3–2.0)
Many	15 (58)	5 (19)	6 (23)	1.5	(0.5–4.5)	6.6*	(2.1–20.3)
p_{trend}				*0.27*		*0.10*	
Skin propensity to burn							
Tan, no burn	37 (84)	5 (11)	2 (5)	1.0		1.0	
Mild burn, tan	184 (75)	44 (18)	18 (7)	1.9	(0.7–5.3)	1.9	(0.4–8.8)
Severe burn	150 (73)	40 (20)	15 (7)	2.3	(0.8–6.2)	2.2	(0.5–10.3)
p_{trend}				*0.15*		*0.34*	
Skin tendency to tan							
Dark tan	73 (79)	13 (14)	6 (7)	1.0		1.0	
Moderate tan	158 (73)	41 (19)	17 (8)	1.5	(0.7–2.9)	1.3	(0.5–3.5)
Mild/no tan	135 (74)	37 (20)	11 (6)	1.5	(0.8–3.1)	1.0	(0.4–2.8)
p_{trend}				*0.23*		*0.86*	
History of blistering sunburn							
No	386 (76)	80 (18)	29 (7)	1.0		1.0	
Yes	38 (68)	11 (20)	7 (13)	1.2	(0.7–2.0)	1.9	(0.9–4.0)

[1]Counts may not sum to the total number of study subjects due to missing data.–[2]ORs adjusted for study center, sex and age group (modeled as continuous variable).–[3]$OR_{1–10\%}$ = OR for 1–10% of tumor cells positively stained *vs.* <1% positively stained.–[4]$OR_{>10\%}$ = OR for >10% of tumor cells positively stained *vs.* <1% positively stained.–*$p < 0.05$.

to have *in situ* melanoma and excluded. Of all 533 tissue samples obtained from the 3 study centers, 523 (98%) had tumor in the recut sections.

One hundred and thirty-four (26%) of the 523 informative specimens were scored as having >1% of cell nuclei positively stained; 97 had 1–10% positive (weak staining) and 37 had >10% positive (strong staining). Rescoring of 42 specimens suggested that the reliability in categorizing tumors into 3 levels (<1%, 1–10%, >10% of cell nuclei stained) was fair (weighted $\kappa = 0.51$, 95% CI 0.29–0.73) (Table I). The κ for the dichotomous score of <1% *vs.* ≥1% (used in the previous study by Whiteman *et al.*[13]) was of a similar magnitude ($\kappa = 0.4$, 95% CI 0.21–0.72), although agreement was higher when specimens were dichotomized at >10% staining ($\kappa = 0.63$, 95% CI 0.26–1.00). There was no disagreement between initial and re-review in distinguishing between specimens with <1% and >10% p53 immunostaining (Table I). Consequently, we present analyses using the 3-level measure of p53 immunostaining with a focus on comparisons between specimens with >10% staining and those with <1% staining as these comparisons are least likely to have been misclassified with each other.

The prevalence of p53 staining differed among study centers (Table II). For Ontario, BC and NSW specimens, respectively, 7%, 11% and 3% demonstrated strong staining. No significant variations in either weak or strong p53 staining were found with age or ethnicity (results not shown), although male sex was positively associated with strong p53 staining (OR = 2.2, 95% CI 1.1–4.8). The probability of both weak and strong p53 staining increased significantly with increasing Breslow thickness. After adjustment for Breslow thickness, strong immunostaining was more prevalent in LMM (OR = 3.2, 95% CI 1.0–10.4) and melanomas of a rare or unclassifiable subtype (OR = 4.8, 95% CI 1.8–12.6) compared to superficial spreading melanoma and less prevalent in melanomas with evidence of a coexisting nevus (OR = 0.3, 95% CI 0.1–0.8).

The relationship between number of melanocytic nevi and p53 immunostaining was inconsistent across measures of nevi (Table III). While increased nevus density as measured by body diagrams was not associated with p53 staining, increased self-reported nevus counts on the back were inversely associated with strong staining ($p_{trend} = 0.01$). Subjects who reported having many facial freckles at age 10 were more likely than those without freckles to have a melanoma with strong staining (OR = 6.6, 95% CI 2.1–20.3, $p_{trend} = 0.10$). The prevalence of strong p53 immunostaining increased with increasing skin propensity to burn (OR = 1.9 and 2.2 for mild and severe burn, respectively), but this trend was not statistically significant. The number of past sunburns was not associated with p53 immunostaining, though a history of blistering sunburn had a nonsignificant positive association with strong p53 staining (OR = 1.9, 95% CI 0.9–4.0). Other measures of skin pigmentation phenotype (skin color, hair color, eye color, tendency to tan) were not associated with p53 staining (data not shown).

Evidence of strong p53 staining was associated with some measures of cumulative sun exposure (Table IV). Melanomas arising on the head and neck (habitually sun-exposed sites) were more likely than trunk melanomas to exhibit strong immunostaining (OR = 2.8, 95% CI 1.2–6.8). Subjects with a previous diagnosis of nonmelanoma skin cancer were more likely than those without to be diagnosed with a melanoma exhibiting strong staining (OR = 2.4, 95% CI 1.1–5.2). The presence of solar elastosis adjacent to the melanoma was associated with strong p53 staining (OR = 2.1, 95% CI 0.9–5.0) but not significantly. Subjects reporting a higher total number of hours of outdoor exposure were also more likely to have a melanoma exhibiting strong staining, although this relationship was not statistically significant ($p_{trend} = 0.40$). When exposures to different patterns of outdoor exposure were separately calculated, high levels of exposure on nonworking days were similarly nonsignificantly associated with strong staining.

TABLE IV – ASSOCIATIONS BETWEEN INDICATORS OF CUMULATIVE SUN EXPOSURE AND EVIDENCE OF P53 IMMUNOSTAINING (1–10%, >10% TUMOR CELL NUCLEI POSITIVE *VS.* <1% POSITIVE) IN CMM (n = 523)

Characteristic	Percentage of cell nuclei positive [n (%)][1]			$OR_{1–10\%}$[2,3]	(95% CI)	$OR_{>10\%}$[2,4]	(95% CI)
	<1%	1–10%	>10%				
Body location of melanoma							
Trunk	182 (77)	37 (16)	17 (7)	1.0		1.0	
Head/neck	41 (66)	11 (18)	10 (16)	1.5	(0.7–3.3)	2.8*	(1.2–6.8)
Upper limb	65 (74)	19 (22)	4 (5)	1.6	(0.8–3.0)	0.8	(0.2–2.5)
Lower limb	93 (74)	27 (21)	6 (5)	1.7	(0.9–3.2)	1.1	(0.4–3.1)
History of nonmelanoma skin cancer							
No	306 (74)	82 (20)	24 (6)	1.0		1.0	
Yes	68 (77)	8 (9)	12 (14)	0.5	(0.2–1.2)	2.4*	(1.1–5.2)
Solar elastosis in adjacent tissue							
Absent	202 (73)	63 (23)	11 (4)	1.0		1.0	
Present	162 (76)	32 (15)	19 (9)	0.7	(0.4–1.2)	2.1	(0.9–5.0)
Total hours of sun exposure during decade years							
Overall							
0–2,200	100 (78)	24 (19)	5 (4)	1.0		1.0	
2,201–3,600	97 (76)	22 (17)	9 (7)	1.0	(0.5–2.0)	1.7	(0.5–5.3)
3,601–5,400	89 (71)	25 (20)	11 (9)	1.7	(0.8–3.4)	2.1	(0.6–7.0)
>5,400	91 (73)	22 (18)	11 (9)	1.6	(0.7–3.6)	1.8	(0.5–6.7)
p_{trend}				*0.20*		*0.40*	
On nonworking days only							
0–1,100	95 (73)	31 (24)	5 (4)	1.0		1.0	
1,101–1,600	102 (82)	13 (10)	9 (7)	0.4*	(0.2–0.8)	1.4	(0.4–4.4)
1,601–2,300	93 (72)	27 (21)	9 (7)	1.0	(0.5–2.0)	1.5	(0.4–4.9)
>2,300	87 (71)	22 (18)	13 (11)	1.0	(0.5–2.1)	2.2	(0.6–7.5)
p_{trend}				*0.61*		*0.23*	
On working days only							
0–900	103 (81)	18 (14)	6 (5)	1.0		1.0	
901–1,700	94 (76)	18 (15)	12 (10)	1.2	(0.6–2.6)	1.9	(0.7–5.5)
1,701–3,000	86 (67)	34 (27)	8 (6)	3.0*	(1.5–6.1)	1.2	(0.4–3.8)
>3,000	94 (74)	23 (18)	10 (8)	2.3	(1.0–5.1)	1.2	(0.4–4.0)
p_{trend}				*0.01*		*0.96*	
To the melanoma site							
0–800	104 (79)	20 (15)	7 (5)	1.0		1.0	
801–2,000	88 (73)	20 (17)	12 (10)	1.3[5]	(0.6–2.6)	2.4[5]	(0.9–6.6)
2,001–3,800	96 (76)	24 (19)	6 (5)	1.5[5]	(0.8–2.9)	1.4[5]	(0.7–2.9)
>3,800	88 (69)	29 (23)	11 (9)	2.3[5]*	(1.1–4.7)	1.1[5]	(0.3–3.6)
p_{trend}				*0.03*		*0.83*	

[1]Counts may not sum to the total number of study subjects due to missing data.–[2]ORs adjusted for study center, sex and age group (modeled as continuous variable).–[3]$OR_{1–10\%}$ = OR for 1–10% of tumor cells positively stained *vs.* <1% positively stained.–[4]$OR_{>10\%}$ = OR for >10% of tumor cells positively stained *vs.* <1% positively stained.–[5]Also adjusted for body location of melanoma.–*$p < 0.05$.

Measures of exposure on working days and of exposure to the site of the melanoma were positively associated with weak staining only (p_{trend} = 0.01, 03 respectively).

Exclusion of cases with LMM from all previous analyses had no material effect on the results.

The most parsimonious model of the 3-level measure of p53 staining (<1%, 1–10%, >10%), identified from both the forward and backward stepping algorithms, contained terms for Breslow thickness, number of nevi on the back, freckling as a child, histologic subtype and previous diagnosis of nonmelanoma skin cancer (Table V).

Discussion

Our case-only analysis was done to investigate whether etiologic factors for CMM are associated with the presence or absence of p53 immunostaining in melanomas, as suggested by Whiteman *et al.*[13] Approximately 26% of melanomas in our study demonstrated >1% of cells positively stained. This proportion is somewhat higher than that obtained by Whiteman *et al.*[13] (18%) but within the range of reported immunostaining prevalence from other studies using DO-7 MAb (5–34%).[20–23]

There is substantial similarity between our findings and those of Whiteman *et al.*[13] (Table VI). In both studies, tumor p53 staining was greater among histologic subtypes other than superficial spreading melanoma, in melanomas located on the head or neck (compared to trunk), among subjects with a history of nonmelanoma skin cancer and among subjects with few nevi, as assessed by one of 2 measures. Tendency to sunburn was nonsignificantly associated with p53 staining in both studies. Results of the 2 studies differed for freckling (we found p53 staining to be greater in those with substantial freckling of the face as a child, while Whiteman *et al.*[13] found the opposite) and Breslow thickness (p53 staining was strongly associated with Breslow thickness in our study, while Whiteman *et al.*[13] observed no such relationship). Whiteman *et al.*[13] studied only men, in whom we found greater p53 staining than in women. Interestingly, we found LMM, a histologic subtype that typically arises late in life on habitually sun-exposed sites, to be significantly more likely to exhibit p53 staining than superficial spreading melanomas; these subtypes were not included in the Whiteman *et al.*[13] study. We also found p53 staining to be positively associated with evidence of solar elastosis, a histologic marker of chronic sun damage, although this was not statistically significant.

Whiteman *et al.*[13] found the associations with melanoma location, tendency to burn, history of nonmelanoma skin cancer and number of nevi to remain present when these variables were adjusted for simultaneously in a model. Except for number of nevi on the back and history of nonmelanoma skin cancer, the variables retained in our multivariate model were not exactly the same as those of Whiteman *et al.*[13] However, our apparently independent determinants of p53 staining reflect the same pattern: variables indicating accumulated sun exposure are positively associated with presence of staining (with history of freckling and a strong association with LMM in our study substituting for tendency to burn and location of melanoma in Whiteman *et al.*[13]) and number of nevi are negatively associated with staining.

TABLE V – CHARACTERISTICS INDEPENDENTLY ASSOCIATED WITH p53 IMMUNOSTAINING (1–10%, >10% TUMOR CELL NUCLEI POSITIVE *VS.* <1% POSITIVE) IN CMM, SELECTED USING STEPWISE AND BACKWARD ELIMINATION ALGORITHMS

Characteristic	$OR_{1-10\%}$[1,2]	(95% CI)	$OR_{>10\%}$[1,3]	(95% CI)
Study center[4]				
Ontario	1.0		1.0	
BC	1.8	(1.0–3.3)	1.7	(0.6–4.6)
NSW	0.4*	(0.2–0.9)	0.4	(0.1–1.3)
Age (years)[4]				
<40	1.0		1.0	
40–54	0.8	(0.4–1.6)	0.8	(0.2–3.1)
55–69	0.7	(0.3–1.4)	0.9	(0.2–3.6)
70+	0.5	(0.2–1.3)	0.5	(0.1–2.2)
p_{trend}	*0.14*		*0.42*	
Sex[4]				
Female	1.0		1.0	
Male	1.4	(0.8–2.3)	2.8*	(1.2–6.9)
Breslow thickness (mm)				
<0.76	1.0		1.0	
0.76–1.50	1.5	(0.8–2.7)	1.1	(0.4–3.1)
1.51–4.00	4.8*	(2.4–9.7)	1.7	(0.5–5.5)
>4.00	6.1*	(1.6–24.3)	3.9	(0.7–20.8)
p_{trend}	*0.0001*		*0.24*	
Nevi on back				
0–3	1.0		1.0	
4–10	1.4	(0.7–2.8)	1.2	(0.4–3.4)
11–25	0.8	(0.4–1.7)	1.7	(0.6–4.4)
>25	0.7	(0.4–1.5)	0.2*	(0.1–0.9)
p_{trend}	*0.24*		*0.09*	
Facial freckles at age 10				
Few	1.0		1.0	
Moderate	1.4	(0.8–2.4)	0.7	(0.2–1.9)
Many	1.7	(0.5–5.3)	9.0*	(2.3–35.0)
p_{trend}	*0.22*		*0.08*	
Histologic subtype				
SSM	1.0		1.0	
NM	0.7	(0.3–1.6)	1.9	(0.5–6.9)
LMM	1.0	(0.3–3.0)	4.7*	(1.3–16.6)
Other, NOS	0.2*	(0.0–0.8)	6.4*	(2.1–20.0)
History of nonmelanoma skin cancer				
No	1.0		1.0	
Yes	0.5	(0.2–1.2)	2.1	(0.8–5.3)

SSM, superficial spreading melanoma; NM, nodular melanoma; LMM, lentigo maligna melanoma; NOS, not otherwise specified. –[1]ORs adjusted for all variables listed in table.–[2]$OR_{1-10\%}$ = OR for 1–10% of tumor cells positively stained *vs.* <1% positively stained.–[3]$OR_{>10\%}$ = OR for >10% of tumor cells positively stained *vs.* <1% positively stained.–[4]Study center, sex and age group included in final model regardless of *p* value.–*$p < 0.05$.

TABLE VI – COMPARISON OF CASE-ONLY FINDINGS FROM WHITEMAN *ET AL.*[13] WITH CORRESPONDING FINDINGS FROM THE PRESENT STUDY

Variable	Whiteman *et al.*[1,3]		Present study	
	$OR_{>1\%}$[1]	(95% CI)	$OR_{>10\%}$[2]	(95% CI)
Breslow thickness (mm)				
<0.76	1.0		1.0	
0.76–1.50	1.5	(0.5–4.6)	1.1	(0.4–2.7)
1.51–3.00	0.8	(0.2–3.3)	1.7	(0.6–5.1)
>3.00	1.6	(0.3–8.9)	6.4*	(2.3–18.0)
Histologic subtype				
SSM	1.0		1.0	
NM	1.7	(0.5–5.9)	3.0*	(1.1–8.3)
LMM			3.8*	(1.2–12.3)
Other	4.4	(0.8–23.1)		
NOS	1.2	(0.4–3.9)		
Other, NOS			6.3*	(2.6–15.8)
Body location of melanoma				
Trunk	1.0		1.0	
Head/neck	2.7	(0.9–8.6)	2.8*	(1.2–6.8)
Upper limbs	1.4	(0.3–5.9)	0.8	(0.2–2.5)
Lower limbs	2.8	(0.7–11.1)	1.1	(0.4–3.1)
Previously diagnosed NMSC				
0	1.0		1.0	
1+	2.8	(1.0–7.8)	2.4*	(1.1–5.2)
Freckling of face as a child				
Nil	1.0			
Few	0.7	(0.2–2.2)	1.0	
Moderate	0.6	(0.2–2.6)	0.8	(0.3–2.0)
Many	0.5	(0.1–2.5)	6.6*	(2.1–20.3)
Skin propensity to burn[3]				
Always tan/tan, no burn	1.0		1.0	
Burn then tan/mild burn, tan	5.3	(0.6–45.7)	1.9	(0.4–8.8)
Burn then peel/severe burn	6.5	(0.8–52.9)	2.2	(0.5–10.3)
Nevi[4]				
0–1	1.0		1.0	
2–9	0.6	(0.1–2.6)	1.0	(0.3–2.8)
10–24	0.1*	(0.0–0.8)	1.7	(0.6–4.7)
25+	0.2*	(0.0–0.9)	0.2*	(0.1–1.9)

SSM, superficial spreading melanoma; NM, nodular melanoma; LMM, lentigo maligna melanoma; NOS, not otherwise specified. –[1]OR for $\geq$ 1% cell staining, *vs.* <1% staining; unadjusted for other variables.–[2]OR for $\geq$ 10% cell staining, *vs.* <1% staining; adjusted for age group sex study center.–[3]Whiteman *et al.* categories indicated on the left; comparable present study categories on the right. –[4]Nevi counted on left arm, back and shoulders for Whiteman *et al.*, counted on back for present study.–*$p < 0.05$.

When we used the definition of p53 staining (>1% positively stained) used by Whiteman *et al.*,[13] we observed statistically significant associations only for melanoma location on the head and neck, dense freckling as a child, high nevus density on the back and Breslow thickness (data not shown); all of these associations were weaker in magnitude than those found comparing >10% staining with <1% staining. This difference could be due to greater misclassification in our study in distinguishing between tumors with weak staining and those with no staining. Such measurement error would be expected to attenuate observed associations toward the null. Our reliability substudy provided evidence of this pattern.

We considered whether other sources of error might explain aspects of our findings. Although our participation rate was only moderate, we believe it is unlikely that our findings were influenced by selection bias. For bias to be present, the relationship between study participation and evidence of p53 staining would have to differ according to exposure status. This is implausible. Our self-report measures of nevus density are susceptible to measurement error, which might have attenuated estimates of their associations with p53 staining. Self-reported numbers of nevi have been found in intermethod reliability studies to have fair to low agreement with dermatologists' nevus counts.[24–26] Measurement error could, therefore, explain our inconsistent findings on the relationship between nevus density and strong p53 staining.

We expected that melanomas from NSW subjects would have a higher prevalence of p53 staining than those from BC and Ontario, given the higher intensity of ambient UV radiation in NSW; instead, we observed the reverse. This cannot be explained by the restriction of NSW specimens to lesions $\leq$2.00 mm as the difference between study centers remained upon restriction to lesions of this thickness. Differences between the NSW and Canadian cases in the interval between tissue sectioning and staining (median of 17 days for Australian specimens, while Canadian specimens were usually sectioned and stained within a day) and in batching of the sections (all Canadian sections were stained and scored before the Australian specimens were) could have led to the observed difference in staining with country of origin. The study pathologists were aware of this difference in batching of specimens and thus were not effectively blinded to the country of origin. However, such systematic differences in staining cannot explain the observed associations with etiologic factors as all associations were adjusted for study center. In addition, there was no difference in the direction or magnitude of study findings when NSW subjects were omitted from the analysis.

What are p53 staining and its determinants telling us about the pathogenesis of melanoma? As noted above, Whiteman *et al.*[13] interpreted them as suggesting a "divergent pathway" for melanoma, one pathway being characterized by abnormal expression

of the *p53* gene and the other occurring in nevus-prone individuals, perhaps mediated by dysfunction of other tumor-suppressor genes, such as *INK4A*. Before considering these and other possibilities, however, we must ask another question: Could p53 staining in melanoma be due simply to accumulated sun exposure and not indicative at all of the pathogenetic pathway for melanoma?

Simulated solar UV induces p53 expression in normal skin in culture, with a peak at about 24 hr and a return to normal levels at about 48 hr.[27] Similar patterns of expression are seen in *in vivo* studies in human volunteers.[28] Moreover, in biopsies of normal skin adjacent to squamous and basal cell neoplasms and melanocytic nevi, a dispersed pattern of DO-7 immunostaining (described as affecting a small proportion of randomly dispersed cells in the basal layer of the epidermis) was found most frequently in samples from strongly sun-exposed sites (94.7%) and least frequently in samples from nonexposed sites (46.2%). Similar differences were seen for a compact pattern of staining (having an uninterrupted row of at least 10 strongly and uniformly staining nuclei).[29] The dispersed pattern was also more common in skin excised in summer than in skin excised in other seasons,[28] which together with the site distribution suggests that sun exposure is the cause of at least the dispersed pattern of staining. Both we and Whiteman *et al*.[13] found staining of melanomas to be greater on usually exposed (head and neck) than not usually exposed (trunk) body sites. However, when we compared the prevalence of p53 staining by season of melanoma excision, we found no evidence that p53 staining was more common in summer than winter. In addition, our observation that p53 staining was much less in NSW, which has much higher ambient UV, than in Canada is not consistent with a direct and relatively short-term effect of sun exposure in causing the staining. It appears unlikely, therefore, that p53 staining in melanoma is simply a reflection of patients' habitual sun exposure and of no pathogenetic significance.

The increased prevalence of p53 staining among thicker and rare or unclassifiable tumors (often at an advanced stage) suggests that the pathogenetic mechanisms underlying high p53 expression affect the later stages of melanomagenesis. Previous studies have reported a higher prevalence of p53 staining and *TP53* mutation in advanced primary and metastatic melanomas.[14] What are the possibilities for different pathogenetic mechanisms underlying the presence and absence of p53 immunostaining in melanoma? Persistence of p53 expression, as indicated by staining, may indicate that the protein's function is impaired or that there is a downstream disturbance of a p53-mediated pathway. Underlying *TP53* mutations are generally thought to be an unlikely source of impairment of p53 function as they have been reported to be rarely present in uncultured primary melanomas.[14] However, up to 30% of primary lesions may have *TP53* mutations,[30,31] some of which are consistent with an origin in UV-induced DNA damage. Moreover, point mutations have been found in 60% of a small series of melanomas in patients with the XPC variant of xeroderma pigmentosum.[32] Most of these melanomas were LMM and two-thirds of the mutations were CC-to-TT tandem transitions typical of UV causation. We have no data on the presence or absence of *TP53* mutations in the melanomas in this study.

In the absence of an inactivating mutation of *TP53,* abnormal expression of other members of the p53 pathway can lead to p53 inactivation. Hdm2, a critical negative regulator of p53, is overexpressed in approximately 50% of primary melanomas and frequently present in combination with p53 overexpression.[33,34] Polsky *et al*.[35] have argued, however, that since overexpression of hdm2 correlates with improved clinical outcome in melanoma, independently of tumor thickness, it is unlikely that it drives an oncogenic process in melanoma.

If impairment of p53 function contributes to the pathogenesis of melanomas that stain positively for p53, what might underlie the possibly nevus-related pathway of melanomas that do not show p53 staining? Whiteman *et al*.[13] speculated that *INK4A* dysfunction could be a responsible factor. *INK4A* mutations, however, are essentially absent from melanocytic nevi, and they and other causes of loss of *INK4A* are rare in sporadic melanomas.[36] However, a *BRAF* mutation is very common in nevi and melanomas and, when absent, an *NRAS* mutation is often present.[37] In addition, *BRAF* mutations have been found more often in superficial spreading than other types of melanoma[38] and in melanomas from skin that is only intermittently exposed to the sun,[39] features of the postulated nevus-associated pathway to melanoma. In contrast, *NRAS* mutations have been most commonly associated with nodular melanomas or LMMs and location on frequently sun-exposed body sites and observed to be more common in melanomas diagnosed in Australia than in northern or central Europe.[40,41] These contrasting patterns for *BRAF* and *NRAS* weaken the inference that *BRAF* mutation underlies the nevus-associated pathway since *BRAF* and *NRAS* both lie in the *RAS–RAF–MEK–ERK* pathway.

In summary, our results substantially confirm those of Whiteman *et al.,*[13] with respect to the correlates of p53 immunostaining in melanoma and support the concept of different etiologic and pathogenetic pathways to melanoma. While impairment of p53 function and *BRAF* mutation may be components of the pathways indicated, respectively, by the presence and absence of DO-7 staining of p53, much more work is required to characterize the steps in these pathways and the relevance to them of their differing etiologic correlates.

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